Bioanalytical Methods

DOI: 10.1002/anie.201005975

Separation-Free Detection of Biological Molecules Based On Plasmon-Enhanced Fluorescence**

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Fluorescence is the most popular interrogation technique for observing and identifying biological molecules and therefore strategies that enhance it are of fundamental importance to improvements in biomolecular detection. When fluorescent molecules are located in close proximity to noble-metal nanostructures, their emission may be strongly enhanced by interaction with intense plasmon-induced electric fields.[1–10] At large separation distances (> 100 nm) these fields have no effect on fluorescence, but as the separation distance decreases fluorescence is enhanced, reaching a maximum when the fluorophore approaches to within a few nanometers of the metal surface.[11] The most common strategy for identifying biological molecules involves the recognition of one molecule by another. This is characterized by specific binding events that decrease the mean separation distance between complementary pairs of molecules from micrometer to nanometer dimensions. The magnitude of this decrease is similar to that which leads to an increase in plasmon-enhanced fluorescence, and therefore the progress of molecular binding events can be monitored in real-time by associating them with an appropriate metal nanostructure. Here we describe how binding events in homogenous solution can be monitored by associating them with 100 nm gold nanoparticles, and show how this can be used to carry out rapid separation-free assays for proteins and small molecules.

One way to envisage how fluorescence is enhanced by a noble-metal nanostructure is to think of the nanostructure and the fluorescent dye as components of a single dipole.[12,13] In this model the nanostructure acts as an optical antenna that absorbs many more photons than could be absorbed by the isolated fluorescent molecule and therefore the excitation rate is increased. The enhanced electric field surrounding the nanostructure can also increase the rate at which the fluorescent dipole is recycled between the excited and ground states, leading to an increase in emission. The sum of these considerations might suggest that the effect on a fluorescent molecule of being located in close proximity to a noble-metal nanostructure would always be fluorescence enhancement, but the excited state can also be quenched.[14–19] In order to investigate these competing effects we synthesized fluorescent polyethylene glycol (fluorescent PEG; see the Supporting Information) with the structure shown in Figure 1 a, and conjugated it to GNPs of different diameter in the range 5–250 nm. The fluorescent dye (DyLight Fluor 649) is a less expensive alternative to Cy-5, and the disulfide bonds in the pyridyldithiopropionyl (PDP) groups rupture on contact with gold and form dative covalent bonds. Size profiles of GNPs before and after coating with fluorescent PEG (see the Supporting Information) showed that the diameter increased by approximately 3 nm, thus indicating that dye molecules are located within 1.5 nm of the particle surface. There was also a slight bathochromic shift (< 2 nm) in the extinction spectra.

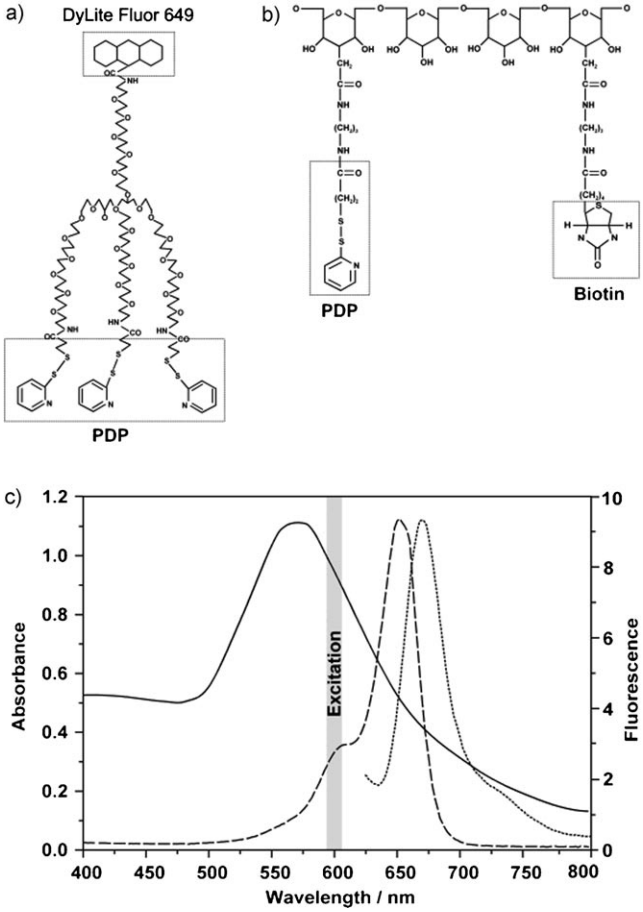

Figure 1. a) Structure of fluorescent PEG; the structure of DyLight Fluor 640 is not in the public domain. b) Structure of biotinylated PDP-dextran; the dextran is a mainly linear polymer with some limited branching; the ratio of PDP to biotin is approximately 1:1 as shown. c) Extinction spectrum of 100 nm GNPs (——), UV/Vis spectrum of fluorescent PEG (– – –), and fluorescence emission spectrum of fluorescent PDP-PEG (•••••).

Figure 1 c shows how the extinction spectrum of 100 nm GNPs overlaps the excitation wavelength (600 nm) and

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[**] This work was supported by a FP7 European project, Bio-Inspired Self-assembled Nano-Enabled Surfaces (BISNES).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201005975.

emission spectrum of the fluorescent PEG. Spectra of the other GNPs used in this study (see the Supporting Information) show that overlap tends to increase as the diameter of the GNPs increases, but it is never absent even at the smallest diameter. Thus, both excitation and emitted light are absorbed by the particles. In addition, light elastically scattered by the GNPs can produce a background signal. In order to accommodate all these variables, emission spectra were first recorded with fluorescent PEG attached to the particles, and then after adding dithiothreitol (DTT) with the particles still present in suspension; DTT releases PDP-PEG from the particles, but has no effect on the spectral properties of the DyLight Fluor 640. Figure 2a shows how the ratio of

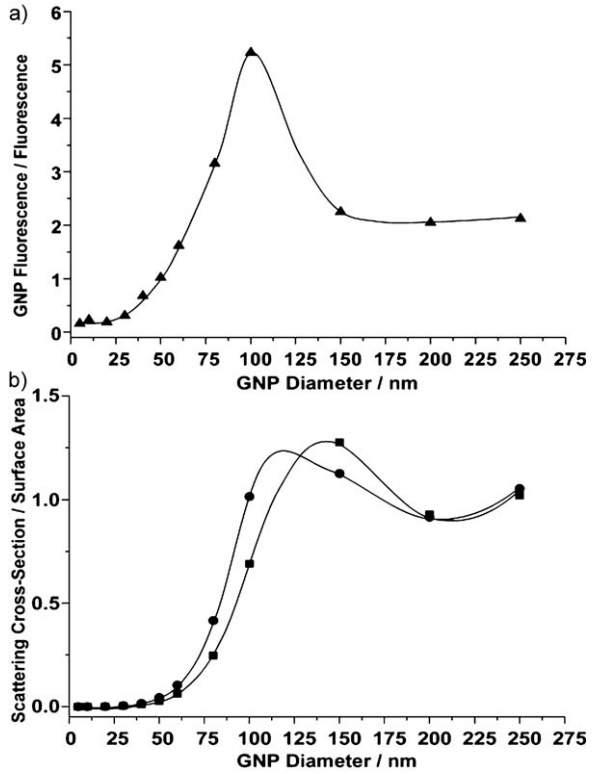

Figure 2. a) Effect of GNP diameter on fluorescence enhancement. Fluorescence enhancement is defined as the fluorescence intensity of fluorescent PEG attached to GNPs divided by the intensity after release from the GNPs, but with the GNPs still present in suspension. b) Effect of GNP diameter on the scattering cross-section of spherical GNPs divided by the surface area at the excitation wavelength of 600 nm (●) and emission peak of 670 nm (■).

fluorescence before and after release of fluorescent PEG from GNPs depends on diameter. Fluorescence is quenched by GNPs with diameters less than 45 nm, but enhanced by larger diameters. GNP-enhanced fluorescence is defined as the emission intensity of a fluorescent molecule in close proximity to a particle divided by the intensity of the free dye molecule. According to the model proposed by Nakamura and Hayashi,[20] enhancement (Y) is described by:

$$Y = [L(\omega_{exc})]^2 Z(\omega_{flu}) \quad (1)$$

where the first term in the equation describes the effect of a GNP on excitation and the second term describes its effect on emission. Both terms depend on the plasmon-induced electric field in close proximity to the GNP, and therefore the dependence on diameter can be estimated by dividing the scattering cross-sections of the particles at the excitation and emission wavelengths by the surface area of the particles as shown in Figure 2b. Qualitative agreement between the experimental and calculated values suggests that this approach is useful for estimating the particle diameter that will produce the greatest enhancement. The main departure of the experimental results in Figure 2a and the model in Figure 2b is at larger diameters. A possible reason for this is that the shapes of GNPs become less spherical as their diameter increases as shown by TEM images in the Supporting Information. This would accentuate anisotropies in the plasmon-induced electric fields surrounding the particles and could lead to a decrease in fluorescence intensity registered by the detector.

Plasmon-enhanced fluorescence decreases exponentially as the distance between a nanostructure and a fluorescent molecule increases. Therefore fluorescence enhancement can be used to report on molecular binding events that lead to the concentration of fluorescent molecules in close proximity to a GNP as shown in Figure 3a. In order to demonstrate this we coated 100 nm GNPs with biotinylated PDP dextran (Figure 1b) and mixed them with fluorescent avidin (avidin labeled with DyLight Fluor 649). Size profiles of GNPs before and after coating with biotinylated dextran showed that the peak diameter increased by approximately 7 nm, thus suggesting that biotin molecules were located within 3.5 nm of the particle surface. Figure 3b shows the difference in fluorescence emission when different amounts of fluorescent avidin are mixed with a fixed amount of biotinylated GNPs in the absence and presence of 10 μM biotin. In the absence of biotin fluorescence is enhanced as avidin molecules bind to the particles, but when biotin is present all binding sites are occupied and binding of avidin to the particles is blocked. Figure 3c shows that fluorescence increases as the ratio of avidin to GNPs increases until it reaches a plateau. Centrifugal washing of the particles showed that the onset of this plateau coincided with the point at which the GNPs were unable to accommodate any more avidin molecules. A single spherical GNP of diameter 100 nm has a surface area of 31 415 nm^2, and avidin has a molecular footprint of approximately 20 nm^2.[21] Therefore each GNP could accommodate approximately 1500 avidin molecules if they were evenly tiled over the surface as a closely packed monolayer, but Figure 3c shows that the actual number of avidin molecules that can be accommodated is greater than this. This is possible because biotinylated dextran increases the surface area available for specific binding. Similar surfaces have been used for many years to enhance the signal in surface plasmon resonance assays,[22] and this result shows that the same strategy can be used to enhance fluorescence signals from nanostructures.

The sensitivity of any detection method depends on the how reproducibly the signal can be distinguished from the background. Maximum sensitivity is achieved when all factors that contribute to nonspecific variations in the signal are

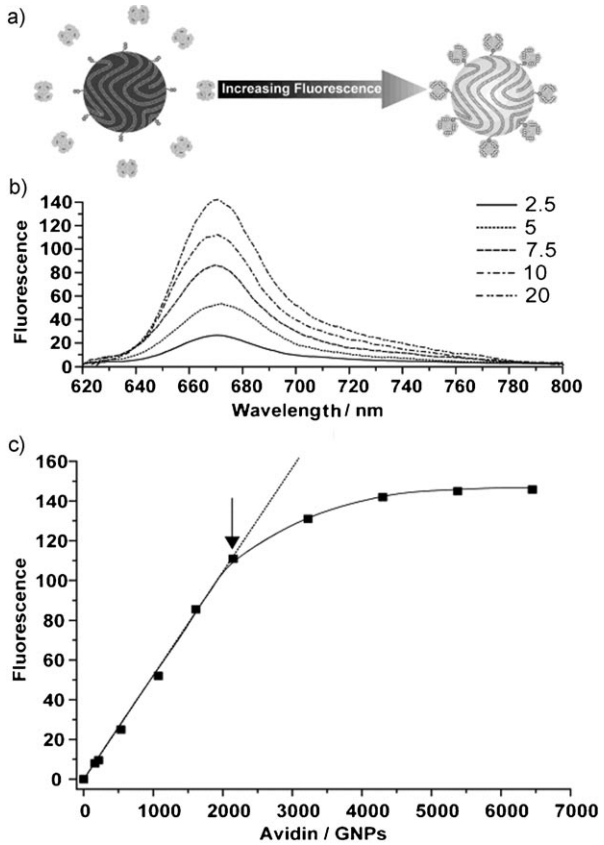

Figure 3. a) Fluorescence is enhanced as avidin molecules labeled with DyLight Fluor 649 accumulate in close proximity to 100 nm GNPs coated with biotin. Although the emission spectrum is identical to that of the fluorescent dye, emission occurs via the particle. b) Emission spectra showing enhancement produced by different amounts of fluorescent avidin added to ca. 2.8×10^9 GNPs (final volume 1 mL) in the absence of biotin; inset key shows the amount of added avidin in pmol. c) Graph showing how the enhancement depends on the ratio of avidin molecules to GNPs.

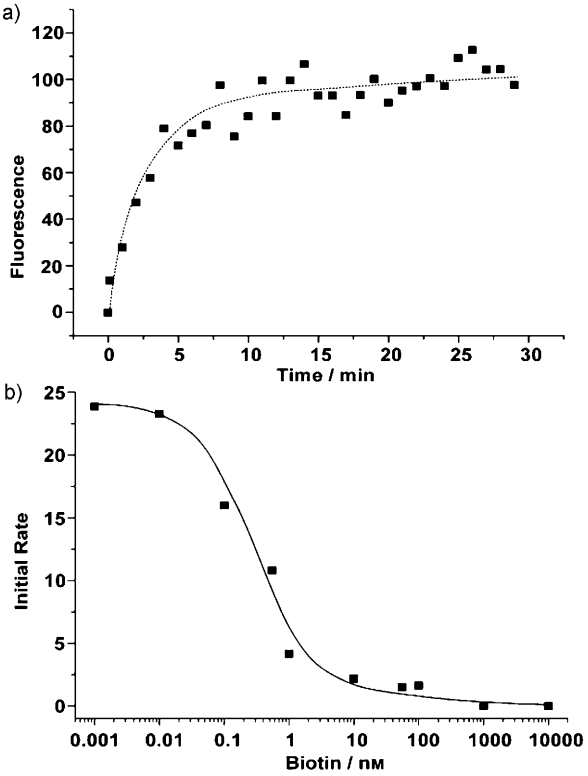

Figure 4. a) Effect on fluorescence of mixing 10 pmol of fluorescent avidin with ca. 2.8×10^9 100 nm GNPs (final volume 1 mL) in the absence of biotin. b) Effect of adding exogenous biotin on the initial rate of fluorescence enhancement.

eliminated. A major source of nonspecific variation in any detection scheme is the error that accumulates during mixing and separation steps, and therefore sensitivity is generally improved by keeping these steps to a minimum. This also facilitates automation and improves accessibility to nonspecialized users with limited training. In order to demonstrate the possibility of carrying out assays that avoid washing and separation steps, we mixed different concentrations of biotin with the ratio of GNPs to fluorescent avidin that corresponds to the point marked by an arrow in Figure 3c. Figure 4a shows how fluorescence enhancement depends on time when fluorescent avidin is mixed with biotin coated GNPs in the absence of biotin. The binding is around 95% complete in 10 min, compared with over 2 h on solid substrates.[10] As the amount of biotin is increased the rate at which fluorescence is enhanced decreases as shown in Figure 4b.

This is the first report describing separation-free assays based on plasmon-enhanced fluorescence in homogenous solution. Szmacinski and co-workers have recently reported a one-step immunoassay based on plasmon-enhanced fluorescence on planar substrates coated with silver islands,[10] but this had much slower kinetics than the solution-phase assay reported here due to mass-transport limitations. Although we have shown how plasmon-enhanced fluorescence in homogenous solution can be used for the rapid detection of avidin and biotin, substitution of antibodies or oligonucleotides for avidin would allow the technique to be extended to a variety of other target molecules. The sensitivity is already comparable to fluorescence quenching assays based on GNPs, but unlike these, binding to the particles in plasmon produces an increase in signal rather than a decrease. This is important because it is easier to detect an increase in signal from a low intensity, than a decrease from a high intensity where the detector must be operated at a lower gain to accommodate the high initial signal. Similarities between enhanced fluorescence and elastic scattering suggest that further improvements in sensitivity could be achieved by using particles with higher ratios of scattering to surface area such as nanoshells.[2] The possibility of using these for separation-free immunoassays based on plasmon-enhanced fluorescence will be the subject of future work.

Received: September 23, 2010
Revised: November 26, 2010
Published online: January 26, 2011

Keywords: nanoparticles · optical antenna · plasmon-enhanced fluorescence · separation-free detection

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